Exhibit D

Efficacy of the Sonicare® Toothbrush Fluid Dynamic Action on Removal of Human Supragingival Plaque

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Abstract

The fluid pressure and shear forces generated by the high frequency bristle motion of the Sonicare® sonic toothbrush remove adherent colonies of cultured bacteria from model dental surfaces *in vitro*. These dynamic fluid effects can remove bacteria *in vitro* even at distances up to 4 mm beyond the tips of the bristles. To evaluate the efficacy of the Sonicare in removing actual human plaque deposits formed *in vivo*, an intraoral model was developed. Enamel sections were obtained from extracted human teeth and mounted on acrylic resin palatal prostheses, worn by two volunteers. Six enamel sections were arranged as three pairs at different locations on the prosthesis, and plaque was allowed to form overnight (~16 h). The sections were removed, placed in phosphate-buffered saline, and exposed *in vitro* to the sonic toothbrush for 5, 10 or 15 seconds. The bristle tips were maintained at distances of 2 or 3 mm from the enamel surface. As a comparison, sections were also exposed to another electric toothbrush (Interplak) for 10 seconds using a distance of 3 mm between the bristles and the enamel. Following exposure to the toothbrushes, residual bacteria were removed from the sections by ultrasonication for 15 seconds, and total viable cell counts determined by serial dilution on blood agar plates. One section from each pair was used to measure total (baseline) microbial accumulation. At a distance of 3 mm between bristles and enamel, the sections exposed to Sonicare demonstrated significant ($p < 0.001$) plaque removal of 56–78% relative to non-treated controls. In contrast, the control electric brush did not demonstrate removal of plaque bacteria after 10 seconds exposure. These quantitative results were visually confirmed by scanning electron microscopy. The findings demonstrate that the fluid dynamic activity generated by the sonic vibrations of the Sonicare toothbrush removed microbial plaque formed *in vivo*, even at a distance of 3 mm beyond its bristle tips. (J Clin Dent 8:10–14, 1997.)

Introduction

Mechanical debridement of teeth by toothbrushing relies on direct contact between the bristles and the tooth surface for the removal of dental plaque. Oral hygiene aids such as interdental brushes, toothpicks and dental floss also rely on direct contact with plaque accumulations to clean the tooth surface. Various oral irrigators project fluids at high velocity into areas where a toothbrush cannot reach, but it is not clear that these are capable of removing plaque,[1-4] and they have been shown in some studies to be unpopular with patients.[5,6] With proper technique, mechanical toothbrushing can consistently remove accessible supragingival plaque, and most studies have reported no significant change in plaque removal with differences in bristle design, texture or brush shape.[7-9] Brushing with electrically powered toothbrushes has been shown to be equivalent to manual brushing in short-term (1–3 month) studies,[10-13] and superior over longer study periods.[12,14]

In addition to cleaning by mechanical scrubbing, the Sonicare® toothbrush (Optiva Corporation, Bellevue, WA) produces fluid pressure and shear forces through high frequency (sonic) bristle vibrations. Previous studies demonstrated that these fluid dynamic effects are capable of removing adherent oral bacteria (*e.g.*, *Streptococcus mutans*, *Actinomyces viscosus* and *Porphyromonas gingivalis*) from dentally relevant surfaces such as titanium and hydroxyapatite disks, even when the bristle tips are at distances up to 4 mm from the substrate surface.[15-17] Furthermore, clinical studies have shown the Sonicare brush to be superior to manual brushing in the removal of supragingival plaque, especially in hard-to-reach areas (*e.g.*, interproximal surfaces).[18,19]

The purpose of the present study was to evaluate the ability of the Sonicare toothbrush to remove naturally formed dental plaque from human tooth enamel, without bristle contact upon the substrate. An *in vivo* intraoral model was developed that permitted the accumulation of plaque microorganisms on human enamel sections. Viable bacteria remaining on the enamel sections were counted following various exposures to the Sonicare toothbrush. In addition, a comparison in ability to remove plaque was made between Sonicare and a conventional electric toothbrush (Interplak®, Bausch & Lomb, Tucker, GA). The present data demonstrate that the fluid dynamic activity generated by the Sonicare device is capable of removing human plaque from enamel surfaces without direct bristle contact. No bacterial removal was seen with the conventional electric brush.

Materials and Methods

Intraoral Model

The intraoral model system consisted of an acrylic resin palatal prosthesis (Figure 1) into which six sections of human molar enamel were affixed with polysiloxane impression material (Reprosil,® LD Caulk, Milford, DE) such that their surfaces were recessed 1 mm into wells created in the acrylic resin (Figure 2). One pair of enamel sections was positioned on the subject's right side (palatal to teeth #3 and #4), one pair palatal to the central incisors (#8 and #9) and the remaining pair was positioned palatal to the maxillary left second premolar and first molar (#13 and #14).

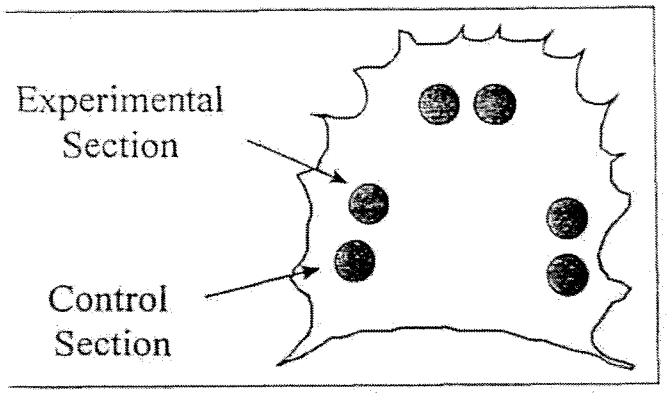

Figure 1. *Palatal prosthesis utilized to collect human plaque samples. The acrylic resin prosthesis was worn overnight (~16 h). After each 16-h period, six samples were collected. These samples were pair-matched based on their location in the prosthesis as described in the Materials and Methods. One sample from each pair was assigned as either an experimental (exposed to fluid dynamic effects of Sonicare) or control section (not exposed).*

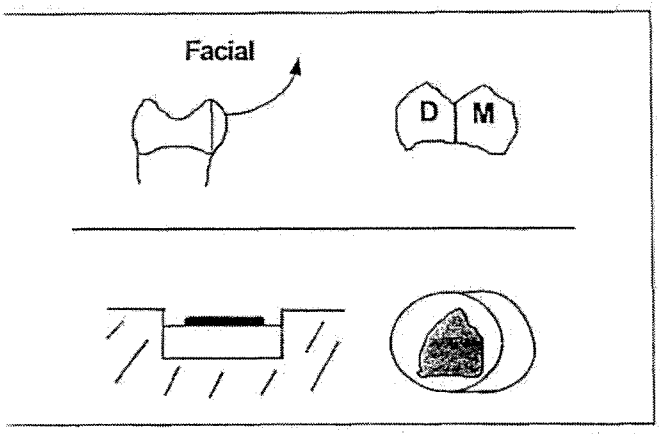

Figure 2. *Top panel: Human enamel sections were made by removing the facial aspect from extracted molars (left drawing). The facial aspect was then sectioned into mesial (M) and distal (D) portions to form two similar enamel sections (right drawing). Bottom panel: Each section was positioned within polysiloxane impression material at the desired depth within a well on the prosthesis (left drawing). After being worn by a volunteer for about 16 h, the prosthesis was removed from the mouth and the impression material, containing the enamel section, was removed intact from the well for further experimentation in vitro (right drawing).*

Preparation of Enamel Sections and In Vivo Plaque Accumulation

Enamel surfaces were obtained from noncarious human molars (Division of Cariology, Dows Institute for Dental Research). All teeth were treated as prescribed by the University of Iowa Institutional Review Board approval (immersion in 10% formalin, 7 ml/tooth, for more than 2 weeks). Samples were prepared by shaping enamel sections (approximate size = 3 × 2 mm) using a diamond bur at high speed, with copious air/water cooling (Figure 2, top panel). Care was taken to avoid adulteration of the test surface of each section. The samples were then ultrasonically cleaned. The exposed surface area (mm^2) of each section was determined by positioning the prosthesis under a calibrated digital camera, capturing the image for each enamel section, and calculating the exposed surface area (Bioquant IV Image System; R&M Biometrics, Inc., Nashville, TN).

All plaque samples in this study were obtained from two human subjects. For each trial, the prosthesis was worn continuously overnight (~16 h) by the subject. Upon returning to the clinic in the morning, the prosthesis was removed, the samples excised and the impression material trimmed flush with the surface of each respective section (Figure 2, bottom panel). The samples were then transfered to sterile phosphate buffered saline (PBS, 0.05M, pH 7.0) until the *in vitro* experimental protocol was performed. This overnight protocol provided three matched pairs/trial/subject and each trial was repeated a minimum of four separate times.

Exposure of Enamel Sections to Sonicare and Quantitation of Plaque Removal

The evaluation of plaque removal was performed *in vitro*. One enamel section of each pair was mounted in a sealed brushing chamber containing 15 ml of sterile PBS. Respective sections were positioned either 2 or 3 mm distant from the bristle tips and exposed to the activated Sonicare toothbrush for 5, 10 or 15 seconds. The remaining section in each pair was placed similarly in the chamber containing PBS for an identical amount of time but without exposure to the device. Following the respective treatments, all enamel sections were placed in individual tubes containing 0.5 ml of sterile PBS and ultrasonicated for 15 seconds (Sonifier® 450, Bronson, Danbury, CT) to remove all adherent bacteria. The viable bacteria released into the PBS from the ultrasonicated enamel sections were determined by serially diluting the PBS and plating the diluents on blood agar plates using a spiral plater (Spiral Systems® Inc., Cincinnati, OH). The plates were incubated at 37°C for 48 h and total viable colony forming units (CFU) enumerated and expressed as the $\log_{10}$ of CFU/mm^2. For each distance and treatment duration, a minimum of 12 pair-matched samples was assessed. The mean value for the treated and non-treated paired samples was calculated for each combination. The mean $\log_{10}$ values were then converted to the numerical equivalent, 10^x, and the percentage plaque removal calculated as:

$$\% \text{ Removal} = 100\% - \frac{\text{Viable CFU per mm}^2 \text{ of exposed enamel section}}{\text{Viable CFU per mm}^2 \text{ of the non-exposed pair matched control}}$$

Exposure of Samples to a Control Toothbrush and Quantitation of Plaque Removal

The ability of a commercially available counter-rotational electric toothbrush (InterPlak) to remove adherent bacterial plaque from enamel sections was examined using identical procedures as described above for a period of 10 seconds at a bristle-to-enamel distance of 3 mm. Quantitation of plaque removal was performed using the same procedures described above. Twenty-nine pair-matched samples were evaluated.

Scanning Electron Microscopy

Samples were fixed for 10 min using 3% v/v glutaraldehyde-formaldehyde in 0.1 M cacodylate buffer, followed by dehydra-

tion with graded acetone and critical-point drying using a CO_2 gas/liquid technique (Balzer CPD 030). Samples were then sputter-coated with 25 nm Au/Pd (Balzer SCD 040) prior to viewing in an Amray 1820D scanning electron microscope (SEM).

Statistical Analysis

Pair-wise comparisons of plaque removal between treated sections and their adjacent non-treated (control) sections were performed. Descriptive statistics of mean viable CFU and standard deviations are reported. Viable microbial counts/unit area were used to calculate ratio percentages of plaque removal, and a one-way ANOVA was performed on the overall differences between the percent plaque removal from Sonicare-exposed samples and non-exposed samples. This was followed by a *post hoc* t-test to compare for differences between groups exposed for varying durations of time and distance. The Tukey-Kramer multiple comparison analysis was used to adjust for multiple comparisons (Prism 2.0, GraphPad, San Diego, CA).

Results

Sufficient and reliably quantifiable amounts of microbial plaque were accumulated on the enamel sections *in vivo* during the 16-h period. The scanning electron micrographs (Figure 3) revealed early plaque formation (after 16 h) exhibiting a majority of coccal cells with occasional rods in a mixed bacterial flora. The ultrasonication procedure was efficient in the complete dislodgment of bacteria from both exposed and unexposed enamel surfaces. The technique for viable cell enumeration provided reproducible results for determination of removal efficiency. The amount of plaque formed was consistent for any one area of the mouth but varied among palatal locations around the oral cavity. The non-exposed section served as a control to normalize total plaque accumulation over the 16-h exposure period.

The Sonicare toothbrush was capable of removing accumulated plaque when the bristle tips were 2 mm or 3 mm distant from the enamel surface. Table I presents the mean values of $\log_{10}$ viable CFU/mm^2 (± SD) on the test and control enamel

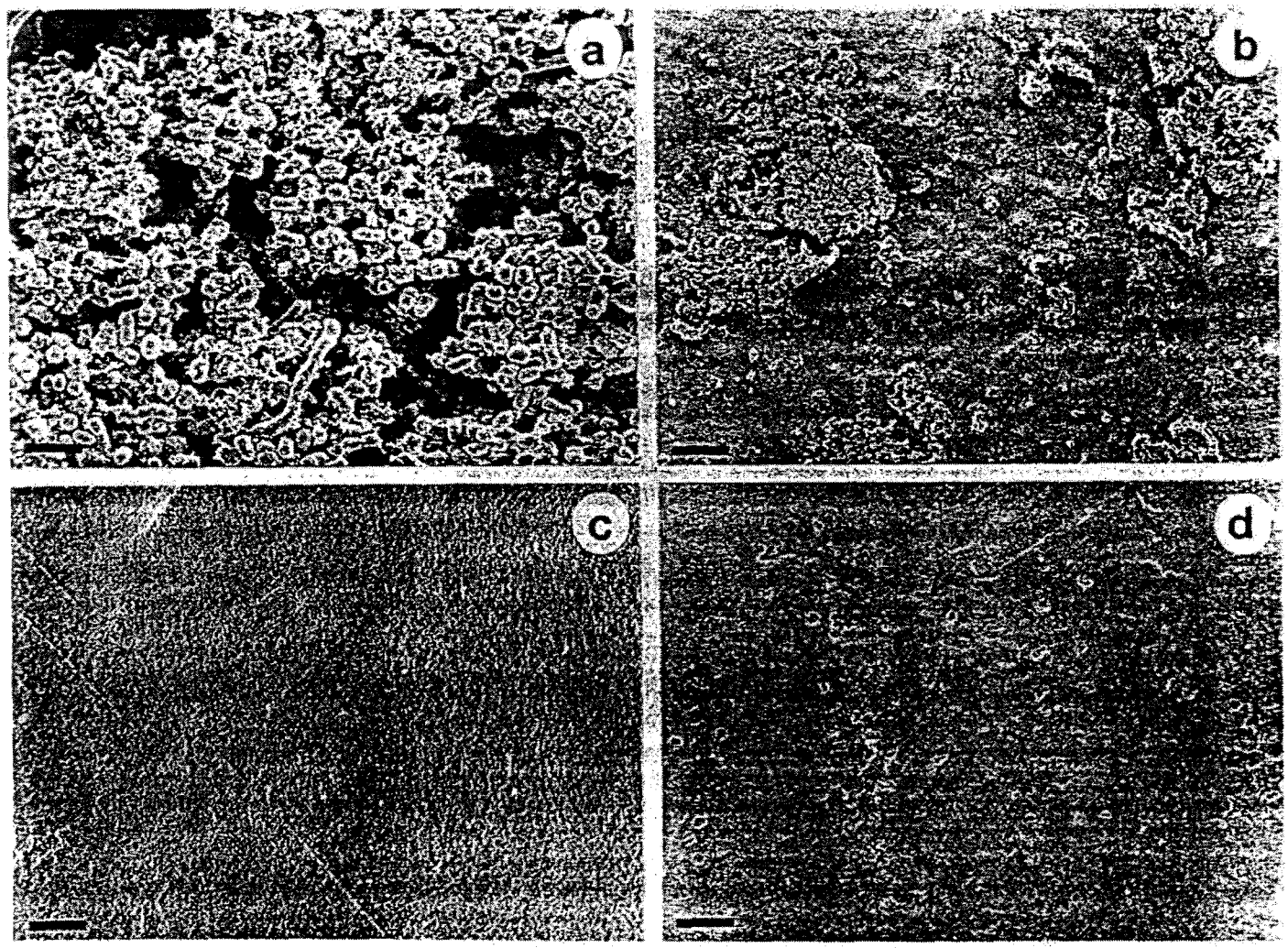

Figure 3. *Scanning electron microscopy of plaque-coated human enamel sections. SEM micrographs were made from plaque-coated enamel sections that were either exposed or not exposed to the fluid dynamic effects of Sonicare. Note the presence of primarily coccal bacterial forms with occasional rods in the mixed microflora (panels a and b). Enamel sections exposed for 15 seconds at a distance of 2 mm from the active bristle tips demonstrate the absence of adherent plaque bacteria (panels c and d). Magnification bar = 2 μm (a and c) and 20 μm (b and d).*

sections. Figure 4 presents comparative percentage plaque removal efficacy data for the Sonicare with bristles in direct contact with the enamel sections (95% regardless of duration of exposure), 2 mm distant from the sections (ca. 65% at all time points), and at 3 mm distance (58%, 63% and 76% at 5, 10 and 15 seconds exposure, respectively). Direct contact with the enamel surfaces for 10 seconds or longer resulted in almost complete removal of viable bacteria (Table I; Figure 4).

Table I
Viable Colony Counts (Mean Log_{10} CFU/mm^2 ± S.D.) Determined on Enamel Sections Exposed and Non-exposed to the Sonicare Toothbrush*

Distance	Treatment	Time (seconds) 5	10	15
Direct Contact	Control†	3.28 ± 1.00	3.20 ± 0.50	3.62 ± 0.60
	Sonicare	0.16 ± 1.00	0.03 ± 0.70	0.04 ± 0.46
2 mm	Control	3.30 ± 1.00	3.20 ± 0.78	3.75 ± 0.80
	Sonicare	2.82 ± 0.85	2.71 ± 0.31	3.26 ± 0.74
3 mm	Control	3.13 ± 0.71	2.96 ± 0.88	3.44 ± 0.61
	Sonicare	2.78 ± 0.61	2.51 ± 0.59	2.78 ± 0.34

* The values represent the means of a minimum of 12 pair-matched samples for each test condition.

† Control specimens were placed in the brushing chamber in PBS for the same amount of time as the Sonicare specimens, but were not exposed to the fluid dynamic effects of the Sonicare.

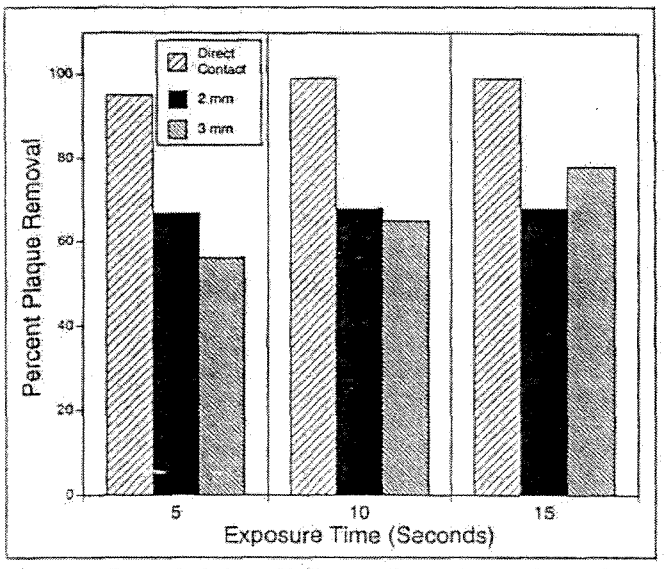

Figure 4. *Removal of plaque biofilm at a distance beyond the bristle tips. Enamel sections positioned at 2-3 mm from the active bristle tips for 5 seconds or more demonstrated 56–78% removal of viable bacteria as compared to non-exposed sections. Direct contact of the bristles produced 95–99% plaque removal. The values shown represent the means from a minimum of 12 pair-matched samples per bar.*

The results of statistical testing of the differences in removal of plaque bacteria from enamel sections exposed or not exposed to Sonicare fluid forces for varying durations of time and distance are shown in Table II. A one-way ANOVA comparing all treatments (various durations of exposure time and distance of

Table II
Omnibus One-Way ANOVA on Percentage Removal of Viable Plaque Bacteria from Enamel Surfaces by Sonicare

Source	Degrees of Freedom	Sum of Squares	Mean Square
Treatment Groups*	8	25675	3209.3
Residuals	99	126.61	1.279
Total Samples	107	25801	

*Groups of samples that are defined by exposure time and distance of bristles from the enamel surface. F = 2509.5; p < 0.0001.

Post-hoc T-Test (Tukey-Kramer Multiple Comparison Test)

Distance	Time (seconds) 5	10	15
Direct Contact	A*	B	B
2 mm	C	C	C
3 mm	D	E	F

*Groups with the same letter (A, B, C, etc.) were not significantly different at p > 0.05 (q < 4.5). Groups with different letters were significantly different from each other at p < 0.001.

bristles from enamel surface) showed that exposure to Sonicare fluid dynamic effects removed highly significant numbers of bacteria ($p < 0.0001$). A *post hoc* t-test comparing the mean percent reduction values from the various groups showed, relative to time, a consistent removal at 2 mm distance. At 3 mm distance, longer exposures (*i.e.*, 10 and 15 seconds) had significantly greater effects.

The scanning electron photomicrographs presented in Figures 3c and 3d reveal that enamel sections exposed to Sonicare at a 2 mm distance exhibited a virtually clean surface. Residual bacteria were observed only in deep pits or in surface defects. Pair-matched control specimens exhibited a confluent layer of microbial plaque (Figures 3a and 3b). Overall, the efficacy of plaque removal decreased as the distance increased from the bristle tips only at the shortest (5 seconds) exposure time.

When enamel samples were prepared in a similar manner and exposed for 10 seconds to the counter-rotational electric toothbrush, with the bristle tips 3 mm from the enamel surface, no plaque removal was detected. The mean log_{10} of CFU as a function of surface area (per mm^2) was 2.92 ± 0.56 for the exposed group and 2.95 ± 0.64 for the brushed group. These values indicate the InterPlak did not remove significant numbers of bacteria from the surfaces of the enamel sections when the bristles were not in contact with the enamel surface.

Discussion

Regular removal of microbial plaque from the dentition is considered an essential component of oral health. The ability to perform this task effectively is often compromised by the patient's dexterity or motivation, by exposed periodontal contours (such as furcations) and/or hard-to-clean prosthetic contours. A mechanically supplemented device, such as an electric toothbrush, facilitates enhanced cleaning efficiency over manual approaches alone.[12,14,18,19] This may be particularly important in special populations such as orthodontic and implant patients, or where the primary oral health regimen must be provided by caregivers. The present study demonstrates that the fluid dynamic action created by the Sonicare toothbrush can lead to an

enhanced removal of viable bacterial flora from human-derived plaque accumulated on human enamel, even when the bristle tips are at some distance from the substrate. This phenomenon is likely related to the sonic frequency movement of the Sonicare bristles creating fluid shear forces capable of disrupting adherent bacterial formations.[15-17]

The ability of a toothbrush to generate an effective dynamic fluid movement at a distance from the bristle tips could lead to enhanced cleaning efficacy. The direct contact obtained with routine oral hygiene approaches can be augmented to reach concavities and other plaque-retentive topographical contours normally missed by conventional electric and manual devices. This may explain, in part, why use of a counter-rotational electric brush was ineffective at plaque removal under the conditions of this study.

The ability to remove plaque under controlled *in vitro* conditions at a clinically relevant distance from the bristle tips suggests that the Sonicare toothbrush may have an additional clinical advantage over conventional manual and electric brushes. Recent clinical studies of periodontitis patients[20,21] and physically handicapped individuals[22] have demonstrated that the use of Sonicare can provide superior oral health. Adult periodontitis patients were found to have generally greater reductions in gingival bleeding, crevicular fluid flow and pocket depth following two-months' use of Sonicare, compared to subjects using a manual toothbrush.[20] Interproximal inflammation in adult periodontitis patients using the Sonicare for six months was reduced by about 50% over that achieved by the Braun Oral-B electric toothbrush.[21] In a study of nursing home residents who required caregivers to provide oral hygiene, the subjects in the Sonicare group exhibited an average plaque reduction of 38% after 6 weeks, compared to a reduction of 6% in the manual brush control subjects.[22] The findings in the present *in vivo/in vitro* study provide further information and new evidence pertinent to one possible mechanism by which superior oral health can be achieved in patients using the Sonicare toothbrush.

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References

1. Lobene RR: The effect of a pulsed water pressure cleansing device on oral health. *J Periodontol* 40:667–670, 1969.
2. Toto PD, Evans CL, Sawinske VJ: Effects of water jet rinse and toothbrushing on oral hygiene. *J Periodontol* 40:296–298, 1969.
3. Lainson PA, Berquist JJ, Fraleigh CM: Clinical evaluation of Pulsar, a new pulsating water pressure cleaning device. *J Periodontol* 41:401–405, 1970.
4. Jackson CL: Comparison between electric toothbrushing and manual toothbrushing with and without oral irrigation, for oral hygiene of orthodontic patients. *Am J Orthod Dentofacial Orthop* 99:15–20, 1991.
5. Lainson PA, Berquist JJ, Fraleigh CM: A longitudinal study of pulsating water pressure cleansing devices. *J Periodontol* 43:444–448, 1972.
6. Peterson WA, Shiller WR: Unsupervised use of a water spray device by naval personnel. *J Periodontol* 39:335–337, 1969.
7. Bay I, Kardel KM, Skougaard MR: Quantitative evaluation of the plaque removing ability of different types of toothbrushes. *J Periodontol* 38:526–533, 1967.
8. Bergenholtz A: Mechanical cleaning. In: *Oral Hygiene*, Frandsen A, Ed., Munksgaard, Copenhagen, pp. 27–62, 1972.
9. Davies AL, *et al*: The effect of variations in toothbrush design on dental plaque scores. *Clin Prev Dent* 10:3–9, 1988.
10. Killoy WJ, *et al*: The effectiveness of a counter-rotary action powered toothbrush and conventional toothbrush on plaque removal and gingival bleeding. A short term study. *J Periodontol* 60:473–477, 1989.
11. Barnes CM, Weatherford TW, Menaker L: A comparison of the Braun Oral-B plaque remover (D5) electric and a manual toothbrush in affecting gingivitis. *J Clin Dent* 4:48–51, 1993.
12. van der Weijden GA, *et al*: The long-term effect of an oscillating/rotating electric toothbrush on gingivitis. *J Clin Periodontol* 21:139–145, 1994.
13. Kambhu PP, Levy SM: An evaluation of the effectiveness of four mechanical plaque-removal devices when used by a trained care-provider. *Special Care Dent* 13:9–14, 1993.
14. Yukna RA, Shaklee RL: Evaluation of a counter-rotational powered brush in patients in supportive periodontal therapy. *J Periodontol* 64:859–864, 1993.
15. Wu-Yuan CD, Anderson RD, McInnes C: Ability of the Sonicare® electronic toothbrush to generate dynamic fluid activity that removes bacteria. *J Clin Dent* 3:89–93, 1994.
16. McInnes C, Engel D, Martin RW: Fimbria damage and removal of adherent bacteria after exposure to acoustic energy. *Oral Microbiol Immunol* 8: 277–282, 1993.
17. McInnes C, *et al*: Reduction in adherence of *Actinomyces viscosus* after exposure to low-frequency acoustic energy. *Oral Microbiol Immunol* 7:171–176, 1992.
18. Johnson BD, McInnes C: Clinical evaluation of the efficacy and safety of a new sonic toothbrush. *J Periodontol* 65:692–697, 1994.
19. Tritten CB, Armitage GC: Comparison of a sonic and a manual toothbrush for efficacy in supragingival plaque removal and reduction of gingivitis. *J Clin Periodontol* 23:641–648, 1996.
20. O'Beirne G, *et al*: Efficacy of a sonic toothbrush on inflammation and probing depth in adult periodontitis. *J Periodontol* 67:900–908, 1996.
21. Robinson P, Maddalozzo D, Breslin S: Clinical comparison of two electric toothbrushes on periodontitis patients. *J Clin Dent* 8:4–9, 1997.
22. Day J, Martin MD: Efficacy of a sonic toothbrush for plaque removal in a special-needs geriatric population. *Special Care Dent*: Submitted, 1996.